

Growth stimulation of the immature chick oviduct by androgens: the vagina as a new target tissue

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SUMMARY

Testosterone when injected alone stimulates growth of the vagina but is inactive upon the other segments of the oviduct of the immature chicken. This action of testosterone can already be detected in embryos: it is expressed by the beginning of differentiation of the vaginal mesenchyme cells into smooth muscle cells. In the treated immature chicken, stimulation of growth is considerable and is specifically caused by androgens (testosterone and 5- α -dihydrotestosterone); the vaginal mesenchyme differentiates into two smooth muscular layers and vaginal epithelium cells differentiate into ciliated cells and goblets cells. [³H]testosterone binding has been found in the vagina of the immature chicken (data not shown). The characteristics of testosterone binding to cytoplasmic components of the chick vagina are consistent with its identity as a testosterone receptor.

INTRODUCTION

The genital tract in the hen is formed from the left oviduct which collects and conveys the ovules released by the ovary into the abdominal cavity. Sexual hormones are present that enable conversion of the embryonic organ, the left Müllerian duct, into the functional oviduct and also control synthesis in the mature oviduct. Hence at the time of sexual maturation weight of the oviduct increases considerably from 0.1 g to 40–60 g (Sturkie, 1954).

Although testosterone is the main androgen steroid hormone, some activity has been shown in the female chicken: growth of the comb and the wattle are dependant on testosterone (Breneman, 1956) and this hormone has been identified in both ovary (Woods & Domm, 1966) and plasma (Etches & Cunningham, 1977) of laying hen. In spite of the fact that a number of studies have been devoted to androgen action on the chick oviduct at different stages of ontogenesis, the

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reported observations and interpretations are somewhat contradictory. In bird embryos the administration of testosterone after sexual differentiation results in either an inhibitory action (Lutz-Ostertag, 1977) or no action at all (Hamilton, 1961) upon the growth of the Müllerian duct.

Results on the immature chicken are even more contradictory and androgens have been shown to have no effect (Palmiter & Haines, 1973; Tokarz, Harrison & Seaver, 1979) very little demonstrable effect (Harrison & Toft, 1973) or a positive effect (Yu & Marquardt, 1973) upon the growth of the oviduct.

The disparity of these observations has prompted us to undertake a more precise study of the action of testosterone on the immature oviduct. This seems warranted especially since it has been clearly established that this hormone acts in synergy with oestradiol (Brant & Nalbandov, 1956) and also that testosterone receptors are present in the oviduct magnum after oestradiol treatment (Harrison & McKenna, 1976; Tokarz *et al.* 1979). It seems that androgens regulate ovomucoid and ovalbumin gene expression independently of oestrogen (Compere, McKnight & Palmiter, 1981).

Here, growth of the oviduct of female embryos and immature chickens has been studied after treatment with testosterone but without primary stimulation or concomitant treatment with oestradiol. Testosterone is found to have a stimulatory effect upon the last segment of the immature oviduct, the vagina.

MATERIALS AND METHODS

Hormones and chemicals

Testosterone heptylate (testosterone), a delayed action androgen which impregnates the organism in a regular and continuous way throughout many days, was obtained from Theramex. 5- α -dihydrotestosterone (5- α -Dht) came from Steraloids Inc. Oestradiol-17 β -benzoate (oestradiol) and progesterone were from Roussel-UCLAF. Tamoxifen was a gift from ICI. All these substances were administered in an oil solution.

Table 1. *Experimental outline*

Experimental group	Age of chickens at autopsy (days)	Number of control female chickens	Number of treated female chickens	Number of injections	Number of days of treatment	Total dose of testosterone (mg)
I	6	10	10	3	4	3.75
II	12	8	8	7	10	8.75
III	19	8	8	12	17	15
IV	25	8	8	16	23	20

The first injection was made on the second day after hatching. The dose injected was 1.25 mg of testosterone heptylate. Animals were killed 24 h after the last injection.

Sources and treatment of birds

White leghorn embryos and Hubbard chickens were purchased from commercial hatcheries. The latter carry a mutation causing copper colouring of female chicks such that they are readily distinguishable from males. Embryos were incubated at 38°C in a humidified incubator and were treated at 10, 12, 14, 16 days after egg laying with 1 mg of testosterone by deposition on the shell membrane after having pierced the shell. 1-day-old birds were injected intramuscularly as indicated in Table 1 of the first experiment. In the second experiment, birds (1-day-old) were treated for 6 days with various hormones (hormones and doses used are indicated in the Figure legends). Control embryos or birds received injections of olive oil in the same amounts as the treated ones. All animals were sacrificed by decapitation 24 h after the last injection.

Biochemical results

At the time of the sacrifice, the oviduct was cut into two parts by a transversal section above the uterus (shell-gland). An anterior part comprising the infundibulum, magnum and isthmus, and a posterior part made up of uterus and vagina were removed.

The reactivity of the five region constituents of the oviduct was not studied separately, the limit between anterior part segments being not clearly visible in the immature oviduct.

Chemical analyses

The oviduct parts were removed and weighed. Nucleic acids were extracted with perchloric acid (Schneider, 1945; Berneman, Lenfant & Lambiotte, 1977). DNA was assayed by the modified diphenylamine reaction (Munro, 1966) using DNA from calf thymus (Sigma) as the standard. RNA was determined by the orcinol method (Ceriotti, 1955) with yeast RNA (Boehringer) as the standard. Proteins were estimated by the modified procedure of Lowry (Markwell, Haas, Bieber & Tolbert, 1978) with bovine serum albumin (BSA) as the standard (Sigma).

DNA synthesis

DNA synthesis was evaluated by measuring the [³H]thymidine incorporation into the anterior and posterior part DNA of the Müllerian ducts of control embryos and of embryos treated with testosterone. After the sacrifice, the anterior and posterior parts were removed and incubated at 37°C in Hanks buffer supplemented with [³H]thymidine ([³H-6]thymidine; specific activity: 27 Ci/mmol; final concentration: 75 nM; C.E.A.). After incubation, the Müllerian ducts were washed at 0°C in Hanks buffer containing non-labelled thymidine (Sigma) at the concentration of 1 mM. The DNA was extracted and dosed according to the technique mentioned earlier. The radioactivity linked to this DNA was measured in a liquid scintillation counter (Intertechnique) with an aqueous counting scintillant (Beckman Ready-solv. GT).

Histological results

The Müllerian duct and the immature oviduct were removed and studied after serial sections.

The left Müllerian duct was fixed with Bouin liquid and embedded in paraffin. The sections (5 µm) were coloured with haematoxylin-eosin or with tetrachrome-Herlant. Observations with the electron microscope were made on organs fixed with glutaraldehyde 2.5% in a cacodylate buffer 0.1 M, then with osmic acid (2%) in the same buffer. After ethanol dehydration, the pieces were embedded in Araldite. The sections were contrasted with uranyl acetate-lead citrate, then observed in the electron microscope (Siemens Elmiskop 101).

RESULTS

*Female embryos*1) *Biometrical analysis*

Amounts of 1 mg of testosterone were administered, since higher doses led to an increase in lethality. Hypertrophy of the right ovary was apparent, testifying to the fact that the hormone was present in the embryos (Hamilton, 1961).

DNA content. No significant variation in DNA content was observed after testosterone treatment of the Müllerian duct either in the anterior or the posterior part (Table 2). The results presented in Table 3 show that no significant increase in DNA synthesis is seen 48 h after hormone treatment. This duration was chosen because it corresponds to the time at which an increase in DNA content, after testosterone treatment, is detectable in the androgen target tissues (prostate and seminal vesicle of castrated rat, Tuohimaa & Niemi, 1966).

Table 2. *Action of testosterone on the anterior and posterior parts of the left Müllerian duct of female embryos: DNA, RNA and protein content*

Contents	DNA μg		RNA μg		Proteins μg	
	AP	PP	AP	PP	AP	PP
Control	11.6 \pm 1.9	14.1 \pm 1.7	14 \pm 1.8	13.4 \pm 1.8	135.5 \pm 11	161.1 \pm 21
1 mg T	10.9 \pm 1.8	13.3 \pm 1.9	14.3 \pm 1.4	22.3 \pm 2.8*	125.5 \pm 13	230.3 \pm 26*

Testosterone (T) was given at a dose of 1 mg per injection at 10, 12, 14 & 16 days of incubation. Autopsy was performed at 18 days. The experiment was carried out on a set of 95 control female embryos and 82 treated female embryos (T). Anterior (AP) and posterior (PP) parts were removed. The results are given as the mean, I.S.E. was made by the T-test.

* $P \leq 0.01$.

Table 3. *Rate of DNA synthesis measured after incorporation of [^3H]thymidine into DNA extracted from the anterior and posterior parts of the left Müllerian duct of 18-day-old control embryos (C) and embryos treated with testosterone (T)*

	Specific activity (cpm/ μg DNA)	
	AP	PP
C	858 \pm 136	956 \pm 203
T	880 \pm 160	998 \pm 185

Treated embryos received 1 mg of testosterone at 16 and 17 days. At 18 days the anterior (AP) and posterior (PP) parts of the left Müllerian ducts were removed and then incubated for 2 h at 37°C with tritiated thymidine. The specific radioactivity of the DNA was then determined as indicated in Materials and Methods.

RNA content. A significant difference ($P \leq 0.01$) in RNA content in the posterior part is seen between treated and non-treated control embryos. One mg of testosterone leads to a 65 % increase. No variation in RNA content is seen in the anterior part however.

Protein content. A 40 % increase in protein content ($P \leq 0.01$) is apparent in the posterior part of the Müllerian duct of embryos treated with 1 mg of testosterone. Protein levels in the anterior part remain similar in experimental and control animals.

These results suggest that the posterior part of the Müllerian duct are slightly sensitive to androgen action, as can be seen by a significant increase in protein and RNA levels, but that the anterior part remains unaffected. It is of interest however that DNA synthesis and hence DNA content is not increased.

2) *Histological analysis*

The five morphologically distinct regions of the future oviduct were examined separately. Only the histological structure of the vagina is modified by the androgen treatment. Light and electron microscopy reveal no change in the epithelium of the vagina, but do show differences in the vaginal mesenchyme. Stimulation of this component, by testosterone, is discrete. Observations with the electron microscope (see Fig. 1) show that many embryonic cells, characterized by a high nucleus/cytoplasmic ratio, still remain, but that some of the cells in the median zone of the mesenchyme have been modified. In control animals (Fig. 1A), these are greatly elongated cells and their cytoplasm is rich in glycogen grains. Unlike those of control embryos which are composed entirely of embryonic cells, in the treated embryos (Fig. 1B) these cells show signs of smooth muscular cell differentiation: nuclei take on a fusiform aspect, an important development of fibrous material with oval bodies, a high glycogen grain content, micropinocytotic invaginations and intercellular contact by nexus, are seen.

Female chickens

1) *Biometrical analysis*

The experimental protocol used to study the effect of testosterone treatment at different times during the development of the immature hen is shown in Table 1. The regional stimulation of growth of only the posterior part of the oviduct can be seen from the results presented in Fig. 2. In the posterior part, fresh weight, DNA, RNA and protein content are already increased after hormonal treatment lasting for 4 days. Hence fresh weight is increased 2.3 times, DNA content 1.3 times and RNA and protein content about twice, when compared with levels in non-treated control chickens. Measurements of these four parameters after continuous administration of testosterone show a steady increase throughout the period studied, and after 23 days growth of the posterior part of the oviduct is radically increased (+400 %) when compared to the anterior part (+30 %).

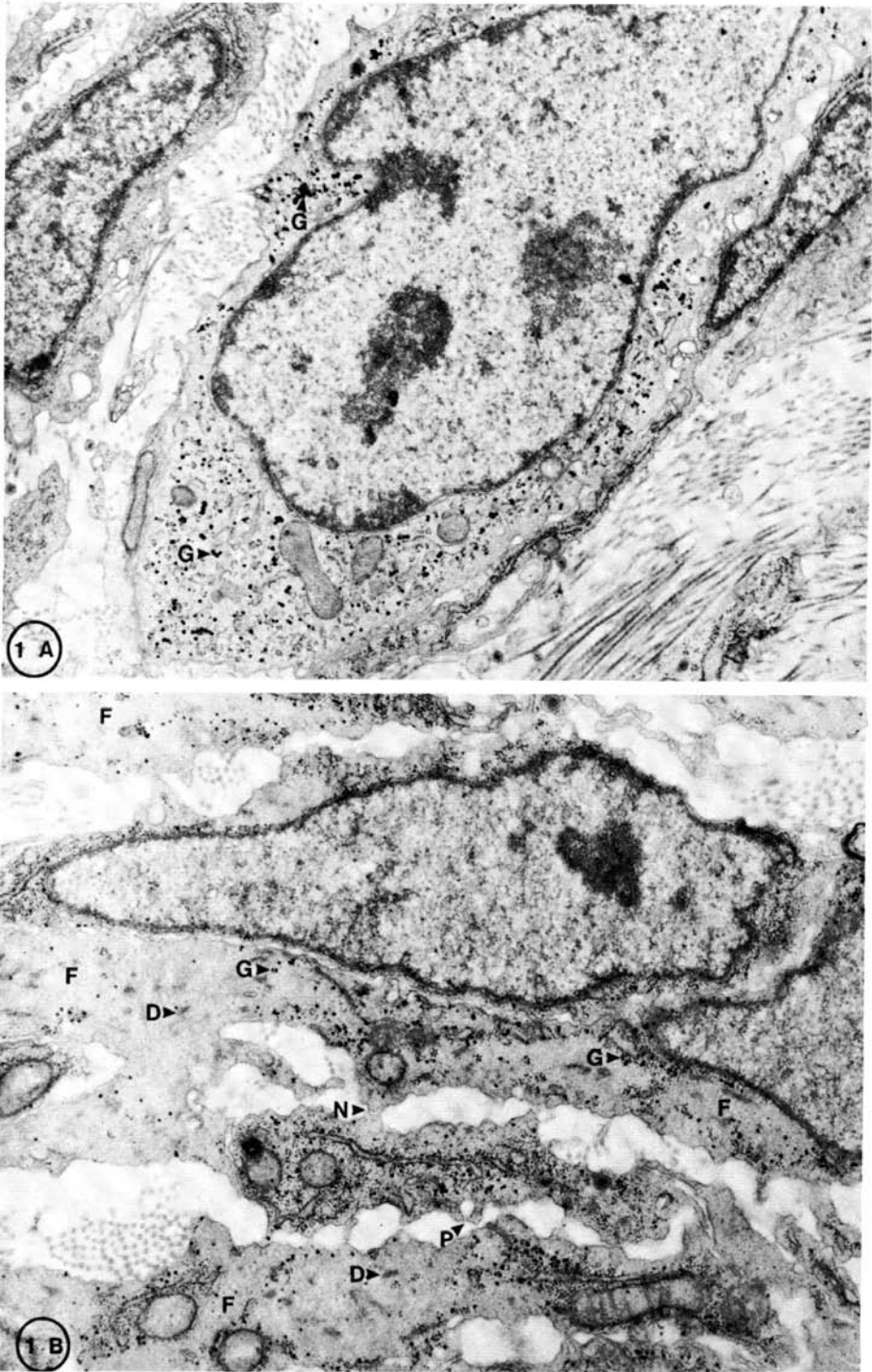


Fig. 1.

Specificity of the action of testosterone. In order to investigate further both the cellular mechanisms and the degree of specificity involved in this testosterone-induced stimulation of growth, a comparison of the effects of different sex steroids has been undertaken (Fig. 3).

No stimulation in the anterior part is recorded by testosterone or its metabolite, 5- α -Dht. Thus, the 5- α -Dht acts like testosterone, selectively on the oviduct posterior part, and in a more significant way. After progesterone treatment, the

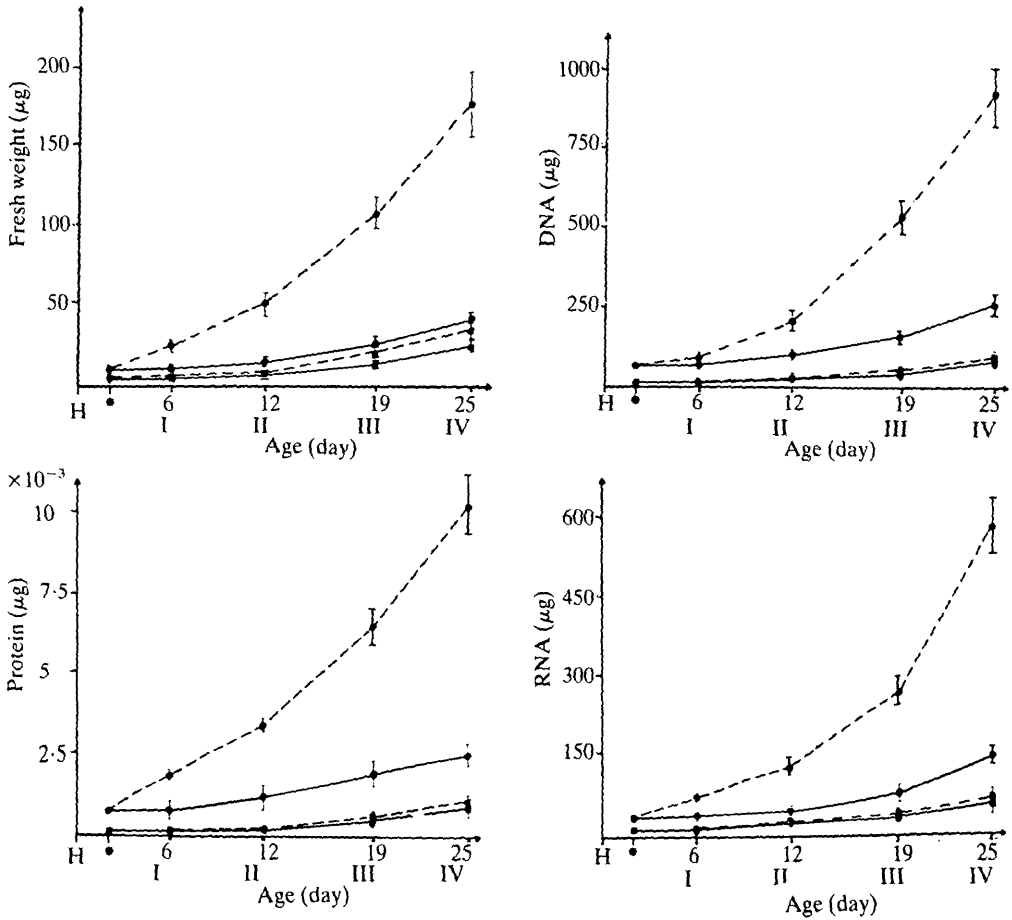


Fig. 2. Levels of fresh weight, DNA, RNA and protein contents in the immature chick oviduct in response to testosterone treatment. Treatment: see Table 1 for treatment designations. H: hatching. * First day of treatment I, II, III, IV. ■—■ anterior part (control); ●—● posterior part (control); ■—■ anterior part (+ testosterone); ●—● posterior part (+ testosterone).

Fig. 1. Smooth muscular cell differentiation in the Müllerian duct vaginal mesenchyme after testosterone treatment. (A) Control embryos ($\times 8000$). (B) treated embryos (four injections of 1 mg testosterone at 10, 12, 14 and 16 days) ($\times 8000$). G, glycogen grain; F, fibrous material; D, oval bodies; N, nexus; P, micropinocytotic invaginations.

protein content and the fresh weight (not shown) are significantly increased only in the anterior part. These parameters do not vary for the posterior part. Oestradiol treatment causes a large increase in the protein content of the anterior and posterior parts so the action of a very powerful anti-oestrogen, tamoxifen, was tested. Tamoxifen does not induce any measurable response in the hen oviduct (Sutherland, Mester & Baulieu, 1977 and Fig. 3). Tamoxifen, which abolishes oestradiol action, does not modify testosterone stimulation of the posterior part.

These observations lead to the conclusion that androgens (testosterone and 5- α -Dht) show a specific action on the posterior part of the immature oviduct. The

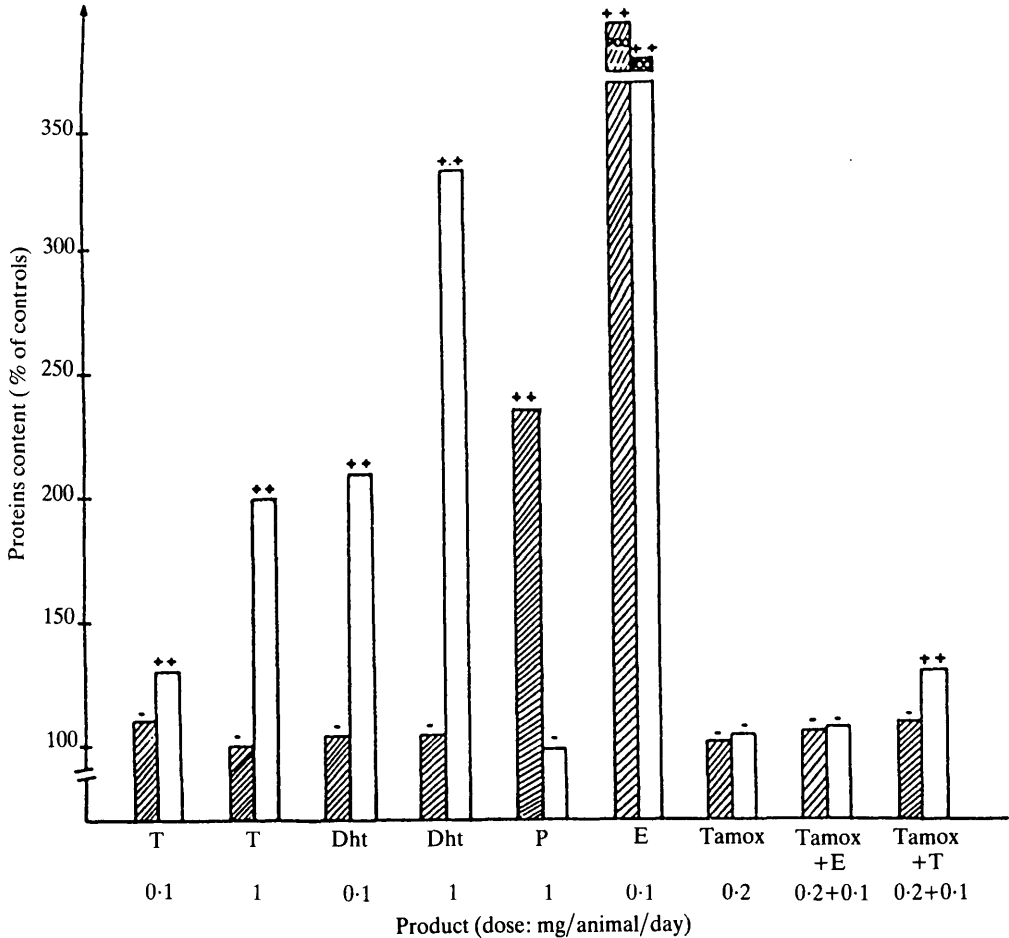


Fig. 3. Protein content in anterior and posterior parts of left Müllerian duct of chicken after different treatments. 24 h after hatching, the pullets were gathered by group of eight and treated daily at the dose indicated of product(s) corresponding during 6 days. Autopsy was made on the 8th day after hatching (24 h after the last injection). The results are expressed in percentage of values found in controls. T, Testosterone; Dht, 5 α -dihydrotestosterone; P, progesterone; E, oestradiol; Tamox, Tamoxifen. * Difference highly significant ($P \leq 0.01$) according to controls. Anterior part equals ▨. Posterior part equals □.

principal metabolite of testosterone, 5- α -Dht, has a stronger effect than testosterone itself.

2) Histological analysis

The analysis was carried out on the five different oviduct regions. Testosterone was given at a dose of 1.25 mg per injection to chicks aged 5 days at the first injection. Three series of androgen treatments were performed at 6, 9 and 13 days.

Anterior part. No appreciable change occurs in the histological structure of the infundibulum, magnum or isthmus after the different period of treatment.

Posterior part. The same conclusion can be drawn from the histological studies of the uterus (Fig. 4A). It is clear that the four anterior oviduct regions are not modified by the hormonal treatment whereas the importance of the histological changes in the vagina is striking (Fig. 4B,C,D) and was to be expected after the histological observation of the same segment in the treated embryos.

Development of the vaginal mesenchyme is considerable (Fig. 4B,D) (formation of the two internal and external smooth muscular layers). During androgen treatment, the thickness of the mesenchyme increases. Luminal epithelium modifications are observed only after 9 days of treatment (cell height and rise in mitotic index, Table 4). Three different classes of cells are visible (Fig. 4C):-

- (i) Ciliated cells, their number increasing during the last treatment.
- (ii) Mucous or goblets cells. Electron microscopy (not shown) revealed an apical cytoplasm full of obvious vacuoles which are thinly striated and faintly electron dense. The free surface of these cells has microvilli.
- (iii) Basal cells, identical to epithelial ones in the control organs, do not present any specialization.

Table 4. Testosterone action on the height and mitotic index of the Müllerian epithelium

		left M.D.		
		Magnum	Uterus	Vagina
Height of luminal epithelium μm	C	8-10	6-10	6-8
	T	8-10	6-10	24-28
Mitotic index ($\times 10^3$)	C	1.1	1.1	1.0
	T	1.1	1.1	3.6

Testosterone was given daily at a dose of 1.25 mg per injection. Age of pullets at the first injection was of 5 days. The duration of treatment was of 9 days. The mitotic index was evaluated by the ratio of the number of cells in mitosis on the total number of cells. C, controls; T, treated.

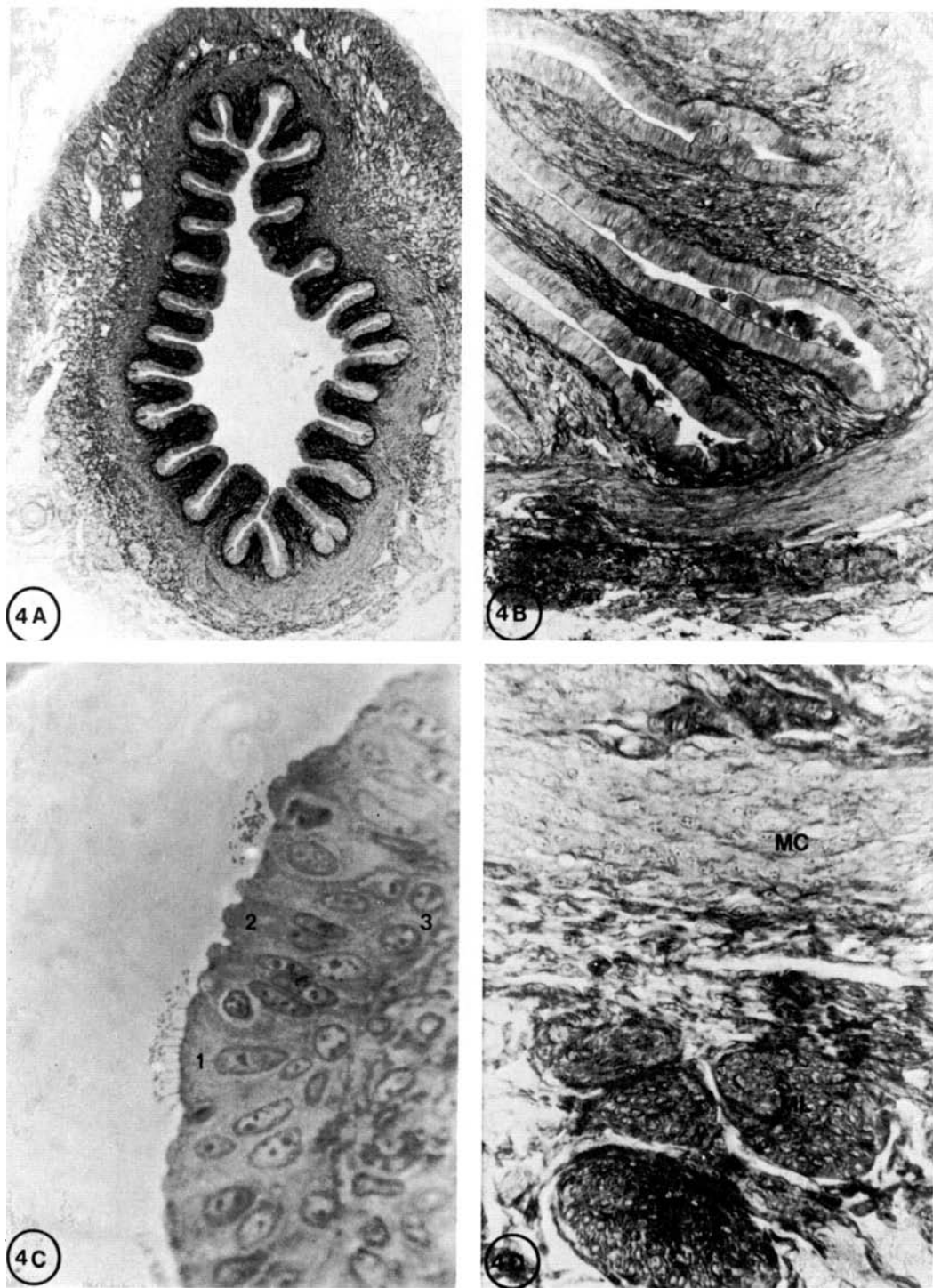


Fig. 4.

DISCUSSION

The present study shows that testosterone, without primary or concomitant treatment with oestradiol, stimulates oviduct growth.

The results show a discrete Müllerian tissue stimulation after testosterone treatment, restricted however to the vaginal part of the embryonic oviduct: RNA and protein contents are significantly increased, but the DNA content does not vary. Similarly Teng & Teng (1979) have demonstrated a partial response of the left Müllerian duct of the chick embryo after oestradiol treatment. Shortly after hatching DNA content is also increased and the vagina attains a higher degree of competence to react to androgen at the end of incubation or shortly after hatching. The acquisition of reactivity to testosterone seems to be, as for oestradiol, a sequential process leading to complete maturation.

After hatching, testosterone shows an important stimulatory effect on the growth of the vagina but is inactive on the other segments of the immature hen oviduct. The existence of oestradiol receptors has been demonstrated in the oviduct of the embryo (Teng & Teng, 1975). So two alternative explanations for testosterone stimulation can be envisaged: either by aromatization of testosterone to oestradiol or by direct action of androgens with receptor systems other than those of testosterone (for example testosterone action of the oestradiol receptors postulated in the immature rat uterus, by Rochefort & Garcia, 1976). Different hormonal and antihormonal treatments demonstrate clearly that stimulation of the immature hen vagina by testosterone is androgen specific. We have shown that progesterone is inactive on the oviduct posterior part. Tamoxifen, which abolishes oestradiol action in the hen oviduct (Sutherland *et al.* 1977), does not modify testosterone stimulation of the posterior part. The hypothesis that stimulation is due to an intermediate stage of aromatization of testosterone to oestradiol or by direct action of testosterone with oestradiol receptor can be ruled out.

Morgan & Wilson (1970) have shown that 5-reductase activity is higher in the uterus than in the magnum of the immature oviduct. However the lack of reactivity of the magnum to testosterone is not explained by this lower enzymatic activity, 5- α -Dht having no effect on the immature oviduct anterior part. These results confirm the conclusions of Tokarz *et al.* (1979) that the androgen hormone has no effect on the immature hen oviduct without preliminary oestradiol treatment. 5- α -Dht turns out to be more active on the vagina than testosterone and its action is very characteristic of some target androgen tissues such as the ventral prostate and the seminal vesicle of the immature rat (Mainwaring, 1977).

Fig. 4. Transversal section of the vagina of immature chick oviduct aged 13 days, as control (Fig. 4A) and treated by testosterone (Fig. 4B,C,D). The androgen treatment was 8×1.25 mg of testosterone. Fig. 4C: 1, ciliated cells; 2, mucous or goblets cells; 3, basal cells. Fig. 4D: MC, internal muscular layers; ML, external muscular layer. Magnification: A, B, $\times 300$; C, $\times 1300$; D, $\times 900$.

The first step of stimulation is the differentiation of the mesenchymatic component, in particular the smooth muscle layers rather than the internal epithelial cells. After 13 days treatment, the young immature hen vagina resembles the functional adult state, both in size and organization (Romanoff & Romanoff, 1949; Gilbert, 1967). Androgen stimulation therefore leads to maturation.

The stimulatory effect of androgen (testosterone and 5- α -Dht) on the immature hen vagina has been demonstrated and part of the active mechanism has been shown. Nevertheless if this segment of the oviduct represents a true target organ for androgen hormones, the presence of receptive molecules specifically binding these hormones must be demonstrated. Further investigations (data not shown) have indicated the presence of testosterone-binding macromolecules in the vagina of the immature chicken (Grau, 1983). [3 H]testosterone binding is organ specific (vagina) and when examined using sucrose gradient centrifugation (Toft & Sherman, 1975), [3 H]testosterone is bound as a 5S and 8S complex. This binding is abolished by a 100-fold excess of unlabelled testosterone but not by similar amounts of oestradiol-17 β or progesterone. These binding properties and the fact that testosterone has been found in immature hen plasma (Etches & Cunningham, 1977) suggest a biological role of testosterone in the oviduct. We can suppose that during the very fast maturation of the left Müllerian duct in the functional oviduct, testosterone would permit the vagina, essentially a muscular organ, to develop as rapidly as the other segments of the oviduct, which are principally glandular and where oestradiol is the primordial hormone (Brant & Nalbandov, 1956).

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